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Phytochemistry 65 (2004) 3131–3134

PHYTOCHEMISTRY

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Flavonoids from shoots, roots and roots exudates of *Brassica alba*

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Received 25 January 2004; received in revised form 7 April 2004
Available online 25 September 2004

Abstract

Analysis of extracts obtained from shoots, roots and exudates of *Brassica alba* revealed the presence of 3,5,6,7,8-pentahydroxy-4′-methoxy flavone in shoots, as well as 2′,3′,4′,5′,6′-pentahydroxy chalcone and 3,5,6,7,8-pentahydroxy flavone in roots and exudates. Apigenin was also found in the shoots and roots, but not in the root exudates.
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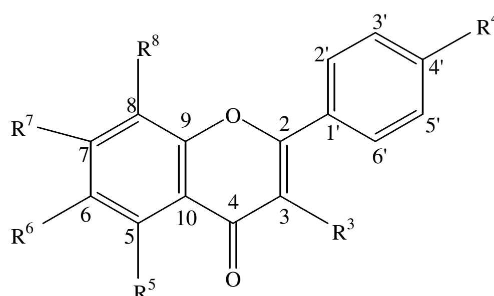
Keywords: *Brassica alba*; Cruciferae; Shoots; Roots; Root exudates; Flavonoids; Flavones; Chalcone; 3,5,6,7,8-Pentahydroxy-4′-methoxy flavone; 2′,3′,4′,5′,6′-Pentahydroxy chalcone; 3,5,6,7,8-Pentahydroxy flavone

1. Introduction

As part of a study of the possible role of flavonoids in symbiotic and parasitic relationships between plants and microbes (Fracchia et al., 2000, 2003, 2004), we decided to isolate and characterize the flavonoids present in shoots, roots and roots exudates of *Brassica alba*. As it is known, *B. alba* shows resistance to mycorrhization. In the present paper, we report the isolation and full characterization of 3,5,6,7,8-pentahydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one or 3,5,6,7,8-pentahydroxy-4′-methoxy flavone (**1**) from shoots and the isolation from roots and exudates of 2′,3′,4′,5′,6′-pentahydroxychalcone (**3**) and a new flavonoid, 3,5,6,7,8-pentahydroxy-2-phenyl-4*H*-1-benzopyran-4-one or 3,5,6,7,8-pentahydroxy flavone (**4**). Apigenin (**2**) was also isolated as the main flavonoid from shoots and roots of *B. alba* but was not detected in root exudates.



Comp.	R^3	R^5	R^6	R^7	R^8	$R^{4'}$
1	OH	OH	OH	OH	OH	OCH_3
2	H	OH	H	OH	H	OH
4	OH	OH	OH	OH	OH	H

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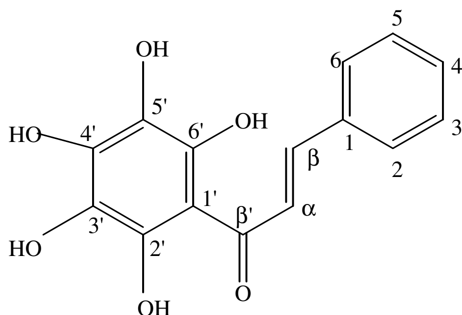
0031-9422/$ - see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.phytochem.2004.08.031

2. Results and discussion

Compound **1** (R_f = 0.88), isolated from shoots of *B. alba* as a colourless solid, showed $[M]^+$ peaks at *m/z* 332 and at *m/z* 332.0530 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_{16}H_{12}O_8$ (*m/z* calc: 332.0532) which was further supported by analysis of its ^{13}C NMR spectrum (Table 1). The IR spectrum displayed absorption bands at 3300–3500 (broad), 1628, 1600 and 1493 cm^{-1}, and compound **1** gave a positive ferric chloride test which indicated that it had free hydroxyl groups (Mabry et al., 1970; Markham, 1982). The ^1H NMR spectrum of **1** showed typical two *para*-coupled doublets with J = 8.15 Hz at δ 7.68 and 6.62, each integrating for two protons, which were assigned to H-2′ and H-6′, and H-3′ and H-5′, respectively. It also showed a sharp three-proton singlet for a methoxyl group at δ 3.75. The EIMS of **1** showed two retro-Diels–Alder fragments at *m/z* 184 (ring-A), 148 ($HOC{=}CC_6H_4OCH_3$, ring-B) and 135 (fragment B_2, $CH_3OC_6H_4CO$) consistent with the presence of the methoxyl group in the ring-B.



Compound **3**.

Compound **3**, R_f = 0.83, was obtained as a yellow solid from roots and exudates of *B. alba*. It gave a molecular ion $[M^+]$ at *m/z* 288 in the EI mass spectrum and at *m/z* 288.0632 in the HREIMS, corresponding to the molecular formula $C_{15}H_{12}O_6$ (*m/z* calc: 288.0634), which was further supported by analysis of its ^{13}C NMR spectrum (Table 1). The IR bands at 3500 (broad), 1653 (C=O) and 1602 (C=C) cm^{-1} and the UV absorption at 354 nm were indicative of a chalcone skeleton with hydroxyl substituents (Mabry et al., 1970; Markham, 1982). The ^1H NMR signals for a set of *trans*-olefinic protons at δ 7.50 and 7.09 (each *d*, J = 15 Hz) confirmed the existence of the chalcone nucleus. Five substituents were attached to the chalcone nucleus, as indicated by signals for two protons at δ 7.73–7.72 (*m*, H-2 and H-6) and for three protons at δ 7.68–7.66 (*m*, H-3, H-4 and H-5) in the ^1H NMR spectrum. The fragment peaks at *m/z* 211 $[M^+ - Ph]$, 185 $[M^+ - PhCH{=}CH]$, 157 $[M^+ - PhCH{=}CHCO]$, 131 $[PhCH{=}CHCO]$, 103 $[PhCH{=}CH]$ in the EIMS of **3** agree with a chalcone structure with an unsubstituted Ph group (Drewes, 1974).

Table 1

^{13}C NMR spectroscopic data (ppm) of compounds **1**, **3** and **4**[a]

C	1	4
2	147.5	147.9
3	137.5	137.8
4	177.0	178.1
4a	96.7	97.1
5	149.6	150.0
6	130.8	130.9
7	144.0	144.7
8	127.6	128.2
8a	152.0	152.2
1′	125.6	129.9
2′	128.1	127.6
3′	113.7	128.7
4′	164.3	130.4
5′	113.7	128.7
6′	128.1	127.6
4′–OMe	55.6	

[a] **3**: 126.4 (C-α), 142.1 (C-β), 192.2 (C=O), 100.6 (C-1′), 152.5 (C-2′), 130.1 (C-3′), 146.4 (C-4′), 130.1 (C-5′), 152.5 (C-6′), 135.1 (C-1), 129.1 (C-2), 128.4 (C-3), 130.4 (C-4), 128.4 (C-5), 129.1 (C-6).

Compound **4** (R_f = 0.62) isolated from roots and exudates of *B. alba* as a colourless solid, showed $[M]^+$ peaks at *m/z* 302 and at *m/z* 302.0425 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_{15}H_{10}O_7$ (*m/z* calc: 302.0427) which was further supported by analysis of its ^{13}C NMR spectrum (Table 1). The IR absorption bands at 3500 (broad), 1627, 1602 and 1490 cm^{-1}, and positive ferric chloride test indicated that compound **4** had free hydroxyl groups (Mabry et al., 1970; Markham, 1982). The ^1H NMR spectrum of **4** showed the two typical *multiplets* of a non-substituted phenyl group integrating for two protons and three protons, respectively. Those signals resonated at δ 7.73–7.71 (*m*, H-2′ and H-6′) and at δ 7.68–7.66 (*m*, H-3′, H-4′ and H-5′). The EIMS of **4** showed two retro-Diels–Alder fragments at *m/z* 184 (ring-A), 101 ($C{=}CC_6H_5$, ring-B) and 105 (fragment B_2, C_6H_5CO) consistent, together with analysis of the ^1H and ^{13}C NMR data, with the presence of the non-substituted phenyl group at C-2 and the hydroxyl group at C-3.

Apigenin (**2**), isolated from shoots and roots, was identified by comparison with an authentic sample and literature data (Agrawal, 1989; The Wiley Registry of Mass Spectral Data, 1996).

Flavonoids found in the shoot and root of many vegetables are hyphotesized to play an important role in the plant–microbe interactions (Vierheilig et al., 1998; Vierheilig and Piché, 2002; Akiyama et al., 2002; Larose et al., 2002) although the necessity of flavonoids for the establishment of AM fungi has been previously questioned by some authors (Becard et al., 1995).

As described elsewhere (Ponce et al., 2004), we did not isolate apigenin (**2**) from *Trifolium repens* with or without AM fungus, so we hypothesize that the resistance of *B. alba* to mycorrhization may be attributed to the presence of this flavonoid. A possible explanation could be found in the toxic effect of apigenin (**2**) on calcium membrane permeability, lowering its concentration and therefore affecting celular turgidity, that is essential in growth by extension (Basile et al., 1999, 2003). Furthermore, apigenin (**2**) inhibits cell proliferation (Boege et al., 1996) and germination of AM spores (Vierheilig et al., 1998). Thus, apigenin (**2**) may protect roots and shoots of *B. alba* to microbial colonization inhibiting its growth inside the plant organs and also might act as a chemical barrier during the first steps of colonization.

The other compounds isolated in this study are two new flavonoids (**1** and **4**) and a new chalcone (**3**), with **3** and **4** being present in roots and root exudates. Regarding the possible role of chalcones in AM symbiosis it had been reported that some chalcones inhibit the hyphal development of the AM fungus, whereas others have been isolated from mycorrhizal and non-mycorrhizal roots (Larose et al., 2002). These results suggest that in this family, as in flavonoid families, the role of a chalcone in the AM symbiosis depends on its structure. Studies in progress in our laboratory (unpublished observations) indicate, that the exuded compounds **3** and **4**, do not affect the germination and hyphal growth of AM fungus, whereas apigenin (**2**) does.

These results, together with the fact that apigenin (**2**) is only present in shoots and roots but is absent in root exudates, can potentially account for an apigenin (**2**) internal chemical role to prevent different infections in *B. alba* without affecting growth of rhizospheric microorganisms and other plants in the surrounding soil. However, whether apigenin (**2**) is the main cause of preventing *Glomus intraradices* colonization still needs to be established.

3. Experimental

3.1. General

UV spectra (Shimadzu UV-1203) were recorded in MeOH, whereas IR spectra (Nicolet 510P FT-IR) were obtained as film on a KBr disc. ^{1}H NMR (Bruker AM-500, 500 MHz) and ^{13}C NMR (Bruker AM-500, 125 MHz) spectra were recorded in DMSO-d_6 with TMS as internal standard, whereas EIMS (Shimadzu QP-5000/Gc-17A/DI-50) and HREIMS (VG-ZAB-VSEQ) were obtained at 70 eV (ionizing potential) using a direct inlet system. Apigenin (**2**) was purchased from Sigma.

3.2. Plant material

Brassica alba plants were grown in 100 ml pots containing steam-sterilized soil mixed 2:1 (v:v) with vermiculite. Plants were grown in a greenhouse, with supplementary light provided by Sylvania incandescent and cold-white lamps, 400 $\mu E\ m^{-2}\ s^{-1}$, 400–700 nm, with a 16/8 h day/light cycle at 25/19 °C and 50% relative humidity, and harvested after 10 weeks with shoots separated from roots. In each case, plant material was dried in an oven at 75 °C for 72 h.

3.3. Extraction and isolation

Dried plant material was crushed and extracted exhaustively with EtOH (98%) at room temperature for 4 days (1.5 l of EtOH per gram of dried material). The ethanol solubles obtained individually from shoots and roots of *B. alba* were filtered and evaporated to dryness (affording syrups, approx. 2.5 g from roots and 16 g from shoots), with each suspended in water (150 ml). The aqueous solutions were then extracted succesively with hexane, EtOAc and CH_2Cl_2 (3×50 ml). All organic fractions so obtained were combined in hexane (defatting), EtOAc and CH_2Cl_2 fractions, and evaporated to dryness with the residues analysed by TLC using precoated silica gel $60F_{254}$ aluminium sheets (0.2 mm thickness, Merck) eluted with EtOAc–CH_2Cl_2–HCO_2H (8:12:1). Chromatograms were visualized after drying (i) by UV light and (ii) by spraying with a solution containing 6 g vanillin and 3 ml H_2SO_4 in 197 ml of MeOH.

The components of the EtOAc extracts (approx. 600 mg from shoots and 150 mg from roots, respectively) were separated by preparative TLC chromatography as above (amount of sample applied <25 mg). After development, the compounds were visualized as above using analytical TLC, then recovered by treating each portion of the adsorbent exhaustively with EtOAc, EtOAc–EtOH and EtOH (3×50 ml), respectively. After evaporation of the combined organic fractions, the main compounds **1**–**4**, were obtained. From 38 g of dried roots, 4.9 mg of **2**, 3.96 mg of **3** and 3.90 mg of **4** were isolated as pure compounds, whereas from 315 g of dried shoots, 40 mg of **1** and 106 mg of **2** were isolated.

Root exudates: The seeds of *B. alba* were sterilized using the following solutions: EtOH:water (1:1) (v/v) 90 s, NaClO solution (2%), 90 s and washed with sterile distilled water several times. Sterile seeds (one per test tube) were scattered in test tubes (3 cm diameter, 30 cm height) with solution ($Ca(NO_3)_2 \cdot 4H_2O$, 0.46 g/l; $MgSO_4$, 0.04 g/l; KH_2PO_4, 0.06 g/l; $Fe_2(SO_4)_3$, 0.00025 g/l; H_3BO_3, 0.0001 g/l and $ZnSO_4$, 0.0001 g/l). After seed germination, the plantlets had their roots submerged in the nutrient solution and the shoots grew to the top of the test tube (8–10 weeks) following which they were harvested and the exudates lyophilized. The residue

obtained (22.1 mg) was extracted with EtOAc, evaporated to dryness and separated by preparative TLC chromatography as detailed before. After evaporation of the organic solvent mixture, the main compounds **3** (3.4 mg) and **4** (2.6 mg) were obtained.

*3.4. 3,5,6,7,8-Pentahydroxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one or 3,5,6,7,8-pentahydroxy-4′-methoxy flavone (**1**)*

$R_f = 0.88$; UV λ_{max}^{MeOH} nm (log ε): 250 (4.43), 308 (4.10); NaOMe: 250, 330. IR ν_{max}^{film} cm^{-1} 3500 (broad), 1628, 1600, 1493. ^1H NMR (500 MHz, DMSO-d_6): δ 7.68 (2H, *d*, $J = 8.15$ Hz, H-2′ and H-6′), 6.62 (*d*, $J = 8.15$ Hz, H-3′ and H-5′), 3.75 (3H, *s*, CH_3O-,C-4′); for ^{13}C NMR (125 MHz, DMSO-d_6), spectral assignments, see Table 1. EIMS 70 eV, *m/z* (%): 332 $[M]^{+\cdot}$ (100), 317 (6), 316 (4), 315 (6), 314 (5), 304 (8), 301 (7), 184 (32), 148 (12), 135 (21); HREIMS *m/z* 332.0530 (calc. for $C_{16}H_{12}O_8$, 332.0532).

*3.5. 2′,3′,4′,5′,6′-Pentahydroxy-chalcone (**3**)*

$R_f = 0.83$; UV λ_{max}^{MeOH} nm (log ε): 354 (4.32). IR ν_{max}^{film} cm^{-1} 3500 (broad), 1653, 1602. ^1H NMR (500 MHz, DMSO-d_6): δ 7.73–7.72 (2H, *m*, H-2 and H-6), 7.68–7.66 (3H, *m*, H-3, H-4 and H-5), 7.50 (^1H (Hβ), *d*, $J = 15$ Hz), 7.09 (1H (Hα), *d*, $J = 15$ Hz); for ^{13}C NMR (125 MHz, DMSO-d_6) spectral assignments, see Table 1. EIMS 70 eV, *m/z* (%): 288 $[M]^{+\cdot}$ (100), 272 (5), 271 (19), 270 (11), 211 (25), 185 (25), 157 (19), 131 (17), 103 (29), 90 (5), 89 (8); HREIMS *m/z* 288.0632 (calc. for $C_{15}H_{12}O_6$, 288.0634).

*3.6. 3,5,6,7,8-Pentahydroxy-2-phenyl-4H-1-benzopyran-4-one or 3,5,6,7,8-pentahydroxy flavone (**4**)*

$R_f = 0.62$; UV λ_{max}^{MeOH} nm (log ε): 354 (4.32). IR ν_{max}^{film} cm^{-1} 3500 (broad), 1653, 1602. ^1H NMR (500 MHz, DMSO-d_6): δ 7.73–7.71 (2H, *m*, H-2′ and H-6′), 7.68–7.66 (3H, *m*, 3H, H-3′, H-4′ and H-5′); for ^{13}C NMR (125 MHz, DMSO-d_6) spectral assignments, see Table 1. EIMS 70 eV, *m/z* (%): 302 $[M]^{+\cdot}$ (100), 286 (3), 285 (4), 274 (8), 184 (23), 105 (45), 101 (7); HREIMS *m/z* 302.0425 (calc. for $C_{15}H_{10}O_7$, 302.0427).

Acknowledgement

We thank UBA (UBACyT X027 and X028) and CONICET (PIP 904/98) for partial financial support. R. Erra-Balsells and A.M. Godeas are research members of CIC (CONICET).

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